Comm.
Dr. Andervont
Dr. Gardner
Dr. Huebner

CARCINOGENESIS

#877R1

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUL 13 1973

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal [X] Second Renewal [ ] Date: July 15, 1973

1. Principal Investigator (give title and degrees): Herbert B. Herscowitz, Ph.D.
Assistant Professor of Microbiology
Schools of Medicine & Dentistry
Georgetown University

2. Institution & address: Schools of Medicine & Dentistry
Georgetown University
37th & "O" Streets, N.W.
Washington, D.C. 20007

3. Department(s) where research will be done or collaboration provided: Department of Microbiology

4. Short title of study: "The Role of the Macrophage in the Immune Response: Effect of Tobacco Products on Macrophage Function."

5. Proposed renewal date: October 1, 1973

6. How results to date have changed earlier specific research aims:

At the time of preparation of this application, I can report that the experimental results obtained to date have not altered our original research aims in any way. We have established a working knowledge of our experimental system and are assuredly approaching the point whereby we will be in a position to test the effects of tobacco products on macrophage function.

7. How results to date have changed earlier working hypothesis:

As specified in our original grant application, we felt that we would require at least the first year of this grant support to establish an appropriate in vitro immunological system as a basis for testing our working hypothesis. We feel that we are making adequate progress toward that goal and will soon be in a position to do the appropriate experiments. Therefore, we do not feel that we have to alter our working hypothesis.

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8. Any additional facilities now required? Describe briefly: The second and third phases of our research plan include a study of the effect of tobacco products on macrophage function and their participiation in the immune response. As will become apparent in reading the proposed experiments, we will have to employ a rather crude system for the exposure of cells and whole animals to cigarette smoke. Having had the opportunity to talk to other investigators, I find that such an approach has severe limitations. We feel that these experiments must be carefully controlled as to smoke volume, duration, intensity and frequency; therefore we request the additional funds to purchase a smoke generating device. We realize that there may be certain strictures on available funds and would hope that the Council would provide us with this instrument either as a purchase item or on loan from another investigator not currently using this device.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

A graduate student, Ms. R. DiBlasio, who had been working on a problem related to this application, has completed her requirements for the degree of Master of Science. She has been replaced by Ms. Kyung Jin Kim whose biographical sketch is attached.

10. Append outline of experimental protocol for ensuing year. See attached.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Two manuscripts are in preparation:

DiBlasio, R., Rosenberg, J.B. and Herscowitz, H.B. A hemolytic plaque assay for the detection of 19S and 7S antibody-forming cells to keyhole limpet hemocyanin (KLH). To be submitted to J. Immunol. Methods.

DiBlasio, R. and Herscowitz, H.B. Preparation and properties of a rabbit anti-mouse macrophage serum. To be submitted to J. Immunol.

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12. Summary progress report (append in standard form as separate document, unless recently submitted). See attached.

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13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Herbert B. Herscowitz, Principal Investigator		REDACTED
Kyung Jin Kim, Graduate Trainee		
Technical		
Jeanette B. Rosenberg		REDACTED
	Sub-Total for A	$14,391
B. Consumable supplies (by major categories)		
1. Disposables and Glassware		1200
2. Tissue Culture Materials		1000
3. Isotopes, Chemicals, Assay Materials		800
4. Animals		1500
5. Miscellaneous		500
	Sub-Total for B	$5,000
C. Other expenses (itemize)		
1. Animal Care		2,000
2. Travel		500
3. Publication		600
4. Equipment Maintenance & Repair		500
	Sub-Total for C	$3,600
	Running Total of A + B + C	$22,991
D. Permanent equipment (itemize)		
1. Smoke Generating Machine		2,500
2. Miscellaneous		1,000
	Sub-Total for D	$3,500
E. Indirect costs (15% of A+B+C)	E	3,449
	Total request	$29,940

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Herbert B. Herscowitz

Signature Herbert B. Herscowitz Date July 15, 1973

Telephone 202 (Area Code) 625 (Number) 7602 (Extension)

Checks payable to

George R. Houston, Jr., Treasurer

Mailing address for checks:

Georgetown University
37th & "O" Streets, N.W.
Washington, D.C. 20007

Responsible officer of institution

Typed Name Mr. Sam A. Kimble

Title Administrator of Sponsored Programs

Signature [signature] Date July 15, 1973

Telephone 202 (Area Code) 625 (Number) 3151 (Extension)

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Justification of Budget

A. Salaries

In accordance with the policy of Georgetown University, I am requesting that R of my salary be paid from this grant. Ms. Jeanette Rosenberg will continue to devote her entire effort toward the experimental goals of this proposal. The values expressed represent an R increase in salary over last year. Fringe benefits have been set at the rate of R by the University.

B. Supplies

These amounts are self-explanatory. Most of our tissue culture will be carried out in disposable lab ware.

C. Other Expenses

An increase in funds is requested in the travel and publication categories over that which was originally requested. The increase in travel is toward the partial cost of attending the Second International Congress of Immunology to be held in Brighton, England during the summer of 1974. I feel that the atmosphere of such a meeting, judging from my experience at the First Congress, will provide up to date information on areas pertinent to this proposal. An increase is also requested for publication costs in view of the fact that we have several manuscripts in preparation.

D. Equipment

An increase in this category is related to the purchase of a smoke generating machine, the justification for which is given in item 8.

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Biographical Sketch

Personal

Name: Kyung Jin Kim
Born:
Citizenship: REDACTED
Marital Status:
Social Security: REDACTED

Education

Sogang University	Seoul, Korea	1969	Biology	B.S.
Louisiana State Univ.	Baton Rouge, La.	1972	Microbiology	M.S.
Georgetown University	Washington, D.C.	--	Microbiology	Ph.D.

Professional Experience

Technical Assistant	Sogang University	1969-1971
Research Assistant	Louisiana State Univ.	1971-1972

Honors

B.S. Degree Cum Laude 1969

Professional Societies

REDACTED

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Research Plan

In view of the possibility that there has been some change in the composition of the Scientific Advisory Board, I will summarily review the background, working hypothesis and goals of our proposed research before outlining the experimental approach to be followed in the ensuing years.

A. Background:

By definition, the basic functional property of a macrophage is its ability to engulf foreign materials. Although they may not demonstrate this capability at all times, the potential for endocytosis is always present. Macrophages take up materials essentially by two closely related processes, phagocytosis (referring to particulate substances) and pinocytosis (usually associated with soluble materials). Both processes are initiated by the interaction of the extracellular foreign material with the cell membrane (1). The particle, after entry into the cell, becomes surrounded by a membrane which eventually closes to form the phagosome. The phagosome detaches and moves to the interior of the cell where it meets and subsequently fuses with a preformed lysosome to form a phagolysosome (2). The hydrolytic enzymes of this secondary lysosome degrade the ingested material and the endocytic function of the macrophage is fulfilled. It is this product of endocytosis which perplexes the immunologist in establishing a functional role of the macrophage in immune induction.

The relevance of phagocytosis to immune processes of host protection is still somewhat uncelar. It has been suggested that there is no correlation between susceptibility to phagocytosis and immunogenicity (3). On the other hand, there is a great deal of evidence which strongly suggests that the macrophage is necessary for the initiation of the immune response (4). Although it was claimed that it is the macrophage that processes the antigen to an immunogenic form and in some cases retains it (5-6), the essentiality of its participation in the induction of the primary immune response was not shown until recently. A requirement for two distinct cell types in the *in vitro* induction of the primary response to SRBC was shown by Mosier (7). In mouse spleen cultures, antibody production appeared to involve both the phagocytosis of antigen by a cell population capable of adhering to glass (macrophage-rich) and antibody production by a non-adherent cell population (lymphocyte rich). Later, this non-adherent cell population was further resolved into its T and B-cell components.

Various methods have been applied toward the separation of lymphoid elements so that studies of cell function could be achieved. Among these methods, was the application of anti-cellular sera to specifically eliminate a single functional cell type. Antisera against partially purified preparations of macrophages have been prepared and tested for immunosuppressive activity (8-10). Unanue (9) found that antimacrophage sera (AMS) caused alterations in both structural and phagocytic characteristics of peritoneal macrophages. Hirsch *et al.* (10) confirmed and extended these findings to show that AMS-treatment resulted in reduced ability of macrophages to adhere to glass and interfered with their ability to phagocytize zymosan particles Further, they showed that treatment of mice with AMS interfered with their ability to resist infection with viral agents, suggesting an altered immune

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mechanism. In extending these experiments to in vivo systems, it was found that pretreatment of mice with AMS resulted in a marked reduction of the primary response to SRBC or diphtheria toxoid (11); however, normal rabbit serum seemed to be as effective in this regard, thereby complicating the interpretation of these experiments. Loewi et al. (12) found that AMS did not inhibit the primary response to SRBC and Unanue (9) failed in an attempt to use AMS to suppress the in vivo response to KLH. On the other hand, it was found that by controlling the dose of immunogen and time of AMS administration the primary response to SRBC could be markedly depressed (13). The work of Jehn et al (14) suggested the necessity of macrophage participation in the secondary immune response. Pierce (15) studied the secondary response to SRBC and suggested that macrophages did not actively participate. On the other hand, Theis and Thorbecke (16) found the SRBC anamnestic response to be macrophage dependent. Using more sophisticated techniques for the preparation and purification of macrophages, Feldmann and Palmer (17) showed that macrophages were required for the induction of the secondary immune response to large complex antigens (i.e. SRBC) but were not involved in the processing of small soluble antigens, suggesting that the requirement for macrophage activity probably depends on the physical size of the antigen. It soon becomes apparent that there is no consistent agreement among investigators as to the requirement for macrophage processing of all antigens to initiate the immune response, however there is compelling evidence that particulate antigens are processed by macrophages for both the primary and secondary responses.

Macrophages also appear to play a role in cell mediated immunity. There is sufficient evidence to suggest that the macrophage functions in the afferent limb of the delayed hypersensitivity response, both by transporting antigen and processing it (18). There have been reports of the successful transfer of tuberculin sensitivity with purified parparations of alveolar macrophages from sensitized animals, suggesting that macrophages can act as effector cells (18). In addition, Thor and Dray (19) have shown that an immunogenic RNA prepared from cells of tuberculin-sensitive donors was capable of transferring delayed reactivity to normal human lymph node cells. There is also evidence to suggest that macrophages contribute to the rejection of allografts. They probably function in the afferent phase of the response by transporting and/or processing antigen. It was found that selective destruction of macrophages or interference with their function prior to grafting resulted in a prolongation of graft survival (20-21). It has also been observed that allografted tumors stimulate an infiltration of macrophages into the tumor area where they contact and kill the tumor cells (22). Further, Old et al. (23) demonstrated that macrophage-rich preparations of peritoneal cells from animals with certain chemically induced tumors could transfer specific tumor immunity to syngeneic recipients. In addition, it has been suggested that non-specific stimulation of macrophages by RES-stimulating agents frequently results in increased resistance to tumors (24).

It is generally accepted that the adaptive immune response evolved as a general vertebrate protective mechanism to eliminate "non-self" components (25). Burnet suggested that the immune system functions as a surveillance mechanism to aid the individual against life-threatening challenge. The surveillance concept has been suggested as a defense in human cancer. In simplest terms, surveillance assumes that tumor cell clones emerge continuously during the

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life of an individual, and that effective mechanisms exist by which the foreigness (tumor clone) is recognized, attacked and destroyed. This can be explained on an immunological basis. There is sufficient evidence to indicate that in the process of transformation to a neoplastic condition, the cell acquires antigens which were not present on the normal parent cell, thus the cell is no longer recognized as self and as a consequence is rendered susceptible to immune attack on the basis of its newly recognized antigenic makeup. One prediction of this theory is that individuals who have immune deficiency, either congenital or acquired, should be subject to a higher incidence of cancer. Indeed, the evidence of Good and Finstad (26) indicates that patients who have congenital immunological defects and patients who have been immunosuppressed following organ transplant develop neoplasms with a greatly increased frequency. Further, in man, the incidence of cancer greatly increases with ensuing age. It has thus been suggested that the development of cancer is associated with a progressive inability to eliminate foreigness which is related to a decrease in efficiency of the immunological defense mechanisms (27). It should be noted that most chemical carcinogens are also immunosuppressive (28) and that many, if not most, viruses known to cause cancer in animals can also suppress the host's immunological reactivity. Thus it would appear that any alteration of the immunological defense mechanism may contribute to the establishment of malignant cells and the eventual demise of the host.

There is sufficient evidence available to indicate that cigarette smoking has an adverse effect on an individual's health. It has been shown that tobacco-derived products are carcinogenic in experimental animals under appropriate conditions. By virtue of their location, alveolar macrophages are of prime importance in protecting the respiratory tract against insult by foreign materials. It is therefore essential that a comprehensive evaluation of the effects of tobacco products on macrophage function be carried out so that we may gain further insight into the effects of smoking on health. However, after a thorough literature search, one finds that there is a paucity of information with regard to the effects of tobacco products on macrophage function.

In this regard, Green and Carolin (29) observed that cigarette smoke depressed the ability of rabbit alveolar macrophages to phagocytize and inactivate S. aureus in an in vitro system. Leuchtenberger and Leuchtenberger (30) compared the effects of charcoal-filtered whole cigarette smoke and unfiltered smoke on the metabolism of mouse and hamster macrophages in vitro. Whereas unfiltered smoke resulted in a series of regressive changes culminating in cell death, the gas phase of charcoal-filtered smoke appeared to enhance metabolic activity as determined by incorporation of ^{3}H-thymidine into DNA. The explanation of this observation is unclear at present, however they suggest that the phagocytic cell may be especially sensitive to the action of cigarette smoke. This is supported by the work of Eichel and Shahrik (31) who reported that charcoal-filtered cigarette smoke inhibited the phagocytic function of human oral leukocytes.

The literature concerning the effect of tobacco products on antibody formation is rather sparse, dated, and confined to in vivo studies. In 1907 Leva (32) studied the effect of injected nicotine on the formation of antibody to the typhus bacillus. In five animals given nicotine (1.5 - 2 mg) daily for

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12-22 days, the immune response was significantly suppressed as compared to the 4 control animals. It should be pointed out that the doses used in these experiments had adverse effects on the animals in that there was convulsion and apathy following nicotine administration. Donzelli (33) acutely exposed immunized animals to cigarette smoke and noted a drop in typhoid agglutinin titer at 6 hours post exposure and which fully recovered in 24-48 hours. In chronically exposed animals, agglutinin titers were reduced to a greater extent than those acutely exposed. Finally in a group of animals immunized during the course of chronic smoke exposure the agglutinin titers were markedly inhibited, the final titer being less than found in unstimulated controls. In another group of experiments, Guardascione (34) found that the injection of nicotine resulted in a reduced response to a heterologous erythrocyte antigen, the decrease being more evident as the dose of nicotine increased. In all of these studies, the effects of tobacco products were evaluated in in vivo systems - one therefore must question whether the immunosuppressive effect observed was due to general protoplasmic poisoning or specific for cells of the immune system.

B. Rationale and Working Hypothesis:

It is postulated that the intact immune system serves as a surveillance mechanism for the inhibition of expression of malignant cell clones. It is apparent that the cells of the reticuloendothelial system are actively involved in maintaining the health of the individual. Numerous studies suggest that macrophages are involved in the induction of both the humoral and cell mediated immune response, probably at the level of antigen processing. There is evidence to indicate that blockade of RES function results in an inhibition of immune responsiveness. Several studies have shown that tobacco products interfere with the phagocytic capabilities of the macrophage. Further, it has been shown that immunosuppression results in an impaired surveillance mechanism so that the patient is rendered more susceptible to insult by pathogenic microorganisms as well as more prone to the development of various forms of malignancy. The question to which we address this research proposal is: Are tobacco products immunosuppressive at the level of the macrophage?

C. Specific Aims:

The specific aims of this research proposal are to study the role of the macrophage in the immune response. We are interested in determining the effects of tobacco products on the ability of macrophages, in general, and alveolar macrophages in particular, to participate in the immune response. Experiments will be devised to clarify and evaluate the following parameters:

(Year 1)

1) To further define the role of the macrophage in the induction of the immune response.

   a) To develop a method for the detection of single antibody forming cells so that we may study cell cooperative effects.
   b) To study the interaction of macrophages with different physical forms of the same immunogen.
   c) To use various methods of cell separation to achieve pure populations of lymphoid cells.

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(1) To prepare and characterize the effects of an anti-macrophage serum on the induction of the immune response.

(Year 2)

2) To determine the effects of tobacco products on the function of macrophages.

a) To study the effect of in vitro and in vivo exposure of peritoneal and alveolar macrophages to nicotine on their phagocytic and other functional capabilities.

(1) To determine the fate and localization of the phagocytized antigen.

b) To study the effect of in vivo and in vitro exposure of peritoneal and alveolar macrophages to cigarette smoke on their phagocytic and other functional capabilities.

(Year 3)

3) To determine the effects of tobacco products on macrophages in relation to their ability to participate in the immune response.

a) To study the effect of these agents on the initiation of both the in vivo and in vitro humoral immune response.

b) To study the effect of these agents on the initiation of the cellular immune response as well as on in vitro correlates of cell-mediated immunity.

D. Experimental Design and Procedures:

1. General Outline

The working hypothesis of this proposal is based on the evidence which suggests that macrophages contribute to the initiation of the immune response at the level of antigen processing and that interference with their phagocytic processes will result in an altered immune response. Although the literature offers numerous references to the participation of macrophages in the immune response, there is considerable controversey as to whether macrophages interact with all forms of antigens in both the primary and secondary immune responses. Therefore, in order to successfully carry out the proposed research regarding the effects of tobacco products on macrophage function, it is first necessary to establish that macrophages are involved in the processing of antigen. Our accomplishments toward that goal as outlined in item 1 of the Specific Aims are given in the attached Progress Report. At the time of this writing, some 3-4 months before the first anniversary of this grant, we feel that we are operating at a pace consistent with the goals set for the first year's accomplishment.

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Once these goals have been achieved, we will study (Year 2) the phagocytic capabilities of macrophages which have been exposed to tobacco products (whole smoke, gas phase and nicotine in vitro as well as the capabilities of macrophages which are derived from animals which have been exposed to tobacco products in vivo. In these studies we will again use the different physical forms of the same immunogen.

In the third phase of this proposal (Year 3) we will study the effects of tobacco products on the ability of macrophages to participate in the initiation of the immune response. Macrophages from tobacco-treated animals or macrophages treated in vitro will be studied for their ability to participate in the initiation of the immune response. Several systems will be employed in these studies. In the first, we will test the ability of tobacco-treated macrophages, after interaction with antigen, to initiate antibody formation in vivo upon transfer of these cells into nonimmune syngeneic animals. In a second system, we will study the ability of tobacco-treated macrophages to initiate the in vitro and in vivo anamnestic responses. And lastly, we will study the effect of tobacco treatment on the ability of macrophages to participate in cell-mediated immune responses.

2. General Procedures:

In view of the fact that our methodological approach has not changed, the procedures to be described in the following sections are essentially the same as submitted in the original application since it was intended to be a three year study. However, there are certain additions to our experimental approach which we feel will add to our basic understanding of macrophage function.

a. Animals and macrophage source:

Two animal systems will be used in these studies. Rabbits will be used as a source of alveolar macrophages for the following reasons: the lung is the prime target of exposure to tobacco products, the rabbit is of sufficient size to yield a workable number of cells, and little is known concerning the contribution of alveolar macrophages to the immune response. The mouse will serve as the source of peritoneal macrophages since a significant amount of data has already been generated concerning the phagocytic capabilities of these cells as well as their participation in the immune response. Although it has been shown that both alveolar and peritoneal macrophages are morphologically similar as to cell type, they appear to exhibit biochemical differences. The hydrolytic enzyme content, which is an important constituent of these phagocytic cells, is 3-4 times greater in alveolar cells than in peritoneal exudate cells (35). The metabolic pathways of the two cell types appear to differ during phagocytosis. Alveolar cells utilize oxidative phosphorylation whereas peritoneal cells appear to utilize the glycolytic pathway for energy (36). In view of these facts we will use both cell types wherever possible so that one will serve as a control for the interpretation of data generated with regard to the other cell type.

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(1) Isolation of Macrophages:

The method of Myrvik et al. (37) will be employed to obtain unstimulated rabbit alveolar macrophages. Briefly, rabbits will be sacrificed by injection of a lethal dose of Diabutal (Diamond Labs, Des Moines, Iowa), the chest cavity opened aseptically and the trachea closed by means of a sterile hemostat. The trachea will be transected above the clamp and the lungs, heart and trachea will be dissected out. A volume of 30 ml. of Hank's balanced salts solution will be injected into the trachea until the lungs are distended, the lungs massaged and the fluid drained into sterile centrifuge tubes. The cells will be recovered by centrifugation at 1,500 rpm for 15 minutes and resuspended in culture medium containing 20% normal rabbit serum. The cell suspension so obtained has been shown to be contaminated by less than 2% of other cell types.

Both stimulated and unstimulated peritoneal macrophages will be obtained from mice. For stimulated peritoneal exudate cells, mice will be injected with 3 ml. of fluid thioglycollate medium (Difco Labs, Detroit, Mich)., according to the procedure of Argyris (38). Three days later, the mice will be sacrificed by exposure to chloroform vapor and 3 ml. of BSS containing 30 units of heparin will be injected into the peritoneal cavity. After gentle massage of the peritoneum, the fluid will be collected and cells recovered by centrifugation as above. The cell pellet will be resuspended in culture medium containing 20% of new-born calf serum. Unstimulated peritoneal cells will be collected by a similar procedure except that fluid thiogylcollate will not be injected.

3. Experiments on the Role of the Macrophage in the Immune Response:

a. General Methods

(1) Antigen:

Keyhole limpet hemocyanin (KLH) which is a potent immunogen, inducing high concentrations of antibody, evoking high levels of immunological memory, and involving a large number of cells in the synthesis of antibody (39) will be used as the antigen. KLH obtained as an ammonium sulfate slurry (Pacific Bio-Marine Supply Co., Venice, Calif)., will be purified according to the procedure developed in my laboratory (40). Briefly, after extensive dialysis the material will be concentrated by ultrafiltration using an XM-100 membrane (Amicon Corp., Lexington, Mass). The KLH will be fractionated on a column of DEAE-cellulose employing a linear salt gradient of 0.05 M NaCl -0.05 M PO_4, pH 6.7 to 0.4 M NaCl - 0.05 M PO_4, pH 6.7. The first peak eluted from the column will be concentrated by ultrafiltration, dialyzed and fractionated by molecular sieving on a column of Bio-gel A 5m (Bio-Rad Labs, Richmond, Calif). The first eluted peak, which is the purified KLH, shows a single component as determined by the following criteria: double diffusion in agar, immuno-electrophoresis, analytical ultracentrifugation, and disc electrophoresis. This material will be adjusted to a final concentration of 2 mg/ml, sterilized by Millipore filtration and stored at 4°C until used.

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(1) Isolation of Lymphocytes

(a) Preparation of dissociated KLH

Purified KLH will be dialyzed against borate-saline buffer, pH 9.0 at 4°C overnight. Since it was found that once dissociated at pH above 8.5, the KLH molecules remain in the dissociated form even after dialysis and extended storage in 0.15 M PBS, pH 7.2 (41); the dissociated KLH will be prepared for use in culture by dialysis against this buffer.

(b) Preparation of aggregated KLH

Purified KLH will be chemically aggregated using the water soluble carbodiimide reagent according to the procedure of Golub et al. (42). In addition, KLH will be rendered particulate by absorption onto bentonite particles (43).

(c) Trace-labelling of KLH

Both associated and dissociated KLH will be trace-labeled with ^{125}Iodine by modification of the chloramine T method as described by Sonada and Schlamowitz (44). We have routinely used this method to obtain labeled proteins of high specific activity.

(2) Tissue culture methods for the initiation of the in vitro immune response

Lymph nodes or spleens from animals previously primed with KLH in vivo will be surgically removed, trimmed of connective tissue and weighed. Cell suspensions will be prepared by disrupting the organ with stainless steel screens. Cells will be washed in 0.15 M PBS, pH 7.2 and adjusted to a final concentration of 2×10^7 nucleated cells/ml. Culture will be carried out in MinimalEssential Medium (Eagle's). The medium will be supplemented with 10% of heat inactivated normal serum (rabbit serum for cultures of rabbit cells and fetal calf serum for mouse cell cultures), 2 mM glutamine, MEM non-essential amino acids, 100 units of penicillin, 50 µg of streptomycin and 1µg of Fungizone as used previously (45). To detect newly synthesized antibody ^{14}C-leucine will be added to the culture medium. Cells will be incubated at 37°C on a roller drum (1 revolution/ 6 min.) in an atmosphere of 5% CO_2 in air.

A portion of the cell suspension will be stimulated with 100 µg of KLH while another portion will be incubated with BSS and will serve as control. After 24 hours, the antigen will be removed from cultures by sedimentation of the cells followed by washing to remove unbound antigen. The culture medium will be changed completely on alternate days. For the determination of newly synthesized antibody, the extra-cellular medium will be dialyzed extensively to remove unincorporated ^{14}C-leucine and subjected to the assay procedure described below. For the determination of cells engaged in antibody formation, cell samples will be removed on various days and assayed by the plaque technique which will be described below.

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(3) Methods for the evaluation of the immune response

(a) Protein and antibody assay

Newly synthesized extracellular proteins will be determined by precipitation of culture fluids with cold 5% TCA as described previously (45). De novo synthesized antibody will be measured by the coprecipitation assay (45) employing ^{14}C-leucine as the precursor of antibody. This method is an extremely sensitive and specific assay of antibody synthesis including antibody of low binding affinity.

(b) Plaque assay

Cells will be assayed for their ability to form hemolytic plaques employing the slide monolayer system of Cunningham and Szenberg (46). Briefly, lymphoid cells at a concentration of 4×10^6 cells/ml. will be mixed with 4×10^8 sheep erythrocytes/ml. which have been coupled with KLH by a modification of the chromic chloride method of Kotoulas and Moroz (47). After the addition of guinea pig complement (1:10), the mixture will be added to each of 3 chambers on a glass slide and incubated for 30 minutes. Plaques will be enumerated by examining the slides under low magnification. To detect indirect plaques (IgG plaques), an appropriate concentration of Anti-IgG serum will be added to the chambers. A full description of the various parameters relating to the establishment of this system as well as kinetic studies employing both rabbit and mouse lymphoid cells are presented in our Progress Report.

b. The Role of the Macrophage in the Immune Response

(1) Preparation of homogenous cell populations

Various procedures are available for the separation and resolution of heterogeneous cell populations into relatively homogeneous cell types. The advantage of using purified cell populations in the study of the cellular interactions in the immune response are obvious if one is to determine the contribution individual cell types to the inductive events of antibody synthesis. Basically, two properties of lymphoid cells have been used to achieve separation, these are based on density differences and the ability of phagocytic cells to adhere to glass.

(a) Separation on the basis of adherence

Cell suspensions will be prepared as described above. The cell suspensions will be incubated in complete MEM at 37°C in plastic petri dishes essentially according to the procedure of Mosier (48). After 2 hours, the supernatant fluid containing non-adherent cells will be removed. The cultures will be incubated overnight and the adherent cell population will be removed with the aid of a rubber policeman.

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(b) Separation on the basis of density

Cells obtained by the described methods will be centrifuged on gradients of bovine serum albumin according to the method of Raidt et al. (49). We will also subject cell populations to density gradient centrifugation on ficoll as described by Gorczynski et al. (50). We have used the latter technique to obtain preparations of macrophages which are 99% pure morphologially.

(c) Treatment of lymphoid cells with carrageenan

Carrageenan, a high molecular weight sulfated polygalactose obtained from marine plants has been shown to be toxic for macrophages both in vitro (51) and in vivo (52). In these studies animals will be injected with varying doses of carrageenan at appropriate times before and after immunization with different physical forms of KLH. Similar experiments will be devised to determine the effect of carrageenan on macrophage function in the in vitro immune responses described herein. These additional studies should further corroborate the functional role of macrophages in antigen processing.

(2) Characteristics of the separated cell populations:

(a) Morphology

The cell populations so obtained will be examined for morphological homogeneity by phase contrast microscopy.

(b) Function

Cells obtained from fractionated gradients will be assayed for functions routinely associated with macrophages. Those fractions determined to have morphological properties of macrophages will be returned to tissue culture and examined for their ability to adhere to plastic. In addition these cells will be incubated with particulate materials and their ability to phagocytize will be evaluated.

(3) Induction of antibody formation

Fractionated cell populations will be tested for their ability to participate in the in vitro immune response. The cell populations will be incubated with the different physical forms of KLH in the presence and absence of macrophages. Cells and culture fluids will be assayed for antibody formation by the methods described. Reconstitution experiments will be performed by adding purified macrophages to the lymphocyte populations followed by in vitro stimulation with KLH. We will also incubate purified macrophages with KLH in vitro and then add these sensitized cells to the lymphocyte population and observe for antibody formation.

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To determine whether previous exposure to KLH by macrophages is required for the induction of the immune response, we will prepare purified alveolar and peritoneal macrophage populations from nonimmune animals or from animals immunized with a different antigen (bovine serum albumin). These macrophages will be added to macrophage-depleted populations of lymphoid cells derived from animals primed with KLH. The magnitude of the observed response should give us an idea as to whether macrophages have a specific function in the antibody response or merely serve as a possible antigen concentrating mechanism in a nonspecific manner.

(4) Macrophage processing of different physical forms of KLH

Purified macrophages will be incubated in suspension culture with ^{125}I-labeled KLH at 37°C according to the procedure of Unanue (53). These experiments will employ the different plysical forms of KLH. The cells will be separated from free antigen, washed and the radioactivity associated with the macrophage will be determined in a gamma spectrometer. To determine the location of the labeled antigen, the macrophages will be disrupted and fractionated by differential centrifugation. To accomplish this, cell extracts will be prepared by disruption of macrophages in a glass Dounce Homogenizer. The homogenate will be centrifuged at 200xg for 5 minutes to remove unbroken cells. The resulting supernatant will be separated into membrane and cytoplasmic constituents by centrifugation. Each of the fractions will be assayed for radioactivity. Such experiments will provide information as to the ability of macrophages to handle the different forms of KLH and will give us some idea as to whether the KLH is processed in the macrophage or if it remains associated with the cell surface. In addition, it will be necessary to determine whether the binding of antigen to macrophages is a function of gamma globulin (cytophilic antibody) or the macrophage surface; to do this we will use heat inactivated serum. Antigen uptake will also be measured in the presence and absence of complement.

(5) Preparation of anti-macrophage serum

An additional approach to the depletion of macrophages from a heterogeneous lymphoid cell population will be through the preparation of a specific anti-macrophage serum. As discussed above, most of these studies performed to date with such a reagent have yielded equivocal results. Macrophages will be obtained from the peritoneal cavity of nonimmune animals as described previously. The cells will be washed and purified by a combination of both of the purification procedures described above. Briefly, cells will be cultured in MEM at 37°C under 5% CO_2. Nonadherent cells will be removed and after a further incubation the adherent cells will be removed and fractionated by density gradient centrifugation on ficoll. Purification will be determined by both morphological and functional criteria. Macrophages so purified will be injected in complete Freund's adjuvant (9) into rabbits by intramuscular and subcutaneous routes. Three weeks later the animals will be given a booster injection of similarly purified macrophages. The animals will be bled at various times and their sera evaluated for specificity and then assayed for antimacrophage activity. Preliminary results of these experiments are in the Progress Report.

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(a) Assay of anti-macrophage activity

Previous reports have shown that serum prepared against mouse macrophages also contained hemagglutinating antibody for mouse red blood cells and showed cytotoxic activity for mouse lymphocytes (9, 13). After assaying our anti-macrophage serum for such activity, appropriate absorptions will be made with mouse RBC and purified suspensions of mouse thymocytes. Finally, we will assay the serum for activity against macrophages by the following criteria: ability to inhibit phagocytic function (9, 14), ability to agglutinate purified macrophage suspensions and ability to injure or kill macrophages in the presence of complement as detected by the release of ^{51}Cr from labeled cells (54). The anti-macrophage serum will then be tested for its effect on the induction of the immune response in two systems. Results of these experiments are in the Progress Report.

(1) In vivo experiments

Both unstimulated KLH-primed mice will be injected with the specific anti-macrophage serum at varying times before and after the injection of antigen in an attempt to achieve macrophage depletion. Control animals will be injected with normal rabbit serum. The animals will be bled at various times thereafter and the sera assayed for circulating antibody levels by passive hemagglutination (55) and by the antigen binding method of Farr (56) as previously described (45).

(2) In vitro experiments

Spleen and lymph node cells from KLH primed animals will be incubated with varying concentrations of specific anti-macrophage serum. The cell population will then be stimulated with KLH and antibody formation determined. Differential cell counts will be made to determine the effective removal of macrophages. Similar cell populations will be treated with normal rabbit serum and serve as controls. In these experiments we will also evaluate the potential handling of the different physical forms of KLH by macrophages.

The experiments described above, constituting the first phase of this proposal, will establish the role of the macrophage in the immune response. Although many of these experiments have been completed and are presented in the Progress Report, the definitive experiments in which we will manipulate the cell population are just at the point where they can be initiated. The above protocols were presented so that new members of the Advisory Board will be familiar with our experimental system.

The remainder of the protocol describes our plans for the next two years.

4. The Effect of Tobacco Products on Macrophage Function

(a) Exposure to tobacco products

In numerous studies attempts were made to simulate actual smoking conditions under experimental situations. However, in evaluating these studies, one must consider that there is tremendous variation among smokers with regard

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to the volume of smoke inhaled with each puff, the number of puffs taken per cigarette as well as the number of cigarettes smoked in a given time period. Therefore, to say that we are simulating actual smoking conditions in experimental situations is somewhat unreal. What we can only hope to do when studying the effects of tobacco products on macrophage function is to use a standardized set of conditions which will be reproducible throughout the experimental design.

We will evaluate the effects of cigarette smoke on macrophage function. In these studies we will use both whole smoke and the gas phase of smoke. Gas phase smoke will be prepared by filtration of whole smoke as described by Leuchtenberger and Leuchtenberger (30). In view of the fact that there are pharmacological differences between components of smoke obtained from the beginning and end of cigarettes, we will use smoke obtained from the middle of a burning cigarette for in vitro studies. Smoke will be collected in a glass syringe fitted with a piece of rubber tubing to hold the cigarette. After determining the volume of smoke obtained from the burning of a whole cigarette, we will use 50 cc of mid-smoke which will be collected in a fresh glass syringe. In order to more rigorously control the variables involved in using such a crude system, we would much prefer to carry-out these experiments with a standardized system such that employing a smoke-generating devise. Therefore, such a machine is requested in our renewal budget.

(1) In vivo exposure to cigarette smoke

Animals from which alveolar macrophages are to be obtained will be exposed to whole smoke or its gas phase for varying periods of time. The animals will be sacrificed and macrophages will be obtained which will be tested for phagocytic function as well as ability to participate in the immune response.

(2) In vitro exposure to cigarette smoke

Macrophages in culture will be exposed to varying volumes of cigarette smoke for varying periods of time according to the procedure of Green and Carolin (29).

(3) Studies with nicotine

In addition to cigarette smoke, we will also study the effect of nicotine on macrophage function. These studies will be included since we can use controlled amounts of a specific component of tobacco. Varying concentrations of nicotine tartrate will be added to cultures of alveolar and peritoneal macrophages according to the procedure of Bond (62).

(b) Experimental Procedures

(1) Effect of tobacco products on phagocytic function

It was observed by Cohn and Ehrenreich (57) that the pinocytosis of certain disaccharides by mouse peritoneal macrophages leads to extensive vacuolization of the macrophage cytoplasm. The vacuoles were shown to be lysosomes containing the pinocytosed disaccharide. Disaccharides which induce vacuolization were

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indigestible and evidently too large to escape readily from the macrophage lysosomes. Thus one has a visible marker to determine the extent of phagocytic function of a cell population.

Pure cultures of alveolar and peritoneal macrophages will be prepared by the methods of adherence and density gradient centrifugation. Cultures will be exposed to smoke and/or nicotine for varying periods of time. Initial experiments will be directed toward the determination of optimal concentration and duration of exposure to tobacco products which will allow for viable macrophage populations. The condition of the cells so treated will be assayed by morphological observation with phase contrast microscopy which will be correlated with cell viability as determined by the trypan blue dye exclusion test. Similar studies will be performed on alveolar macrophages obtained from animals treated with tobacco products in vivo. After exposure to tobacco products, the macrophages will be transferred to fresh media containing KLH bound to bentonite particles and following incubation for varying periods of time, the cells will be fixed in glutaraldehyde and the number of vacuoles in the cytoplasm will be enumerated and compared to untreated controls.

Once that information is obtained, treated macrophage populations will be incubated with ^{125}I-labeled KLH and the uptake, fate and localization of this material will be evaluated as described. In these studies we will employ the various physical forms of KLH. These studies should provide information as to the effect of tobacco products on the phagocytic functions of macrophages.

(2) Comparison of the effects of tobacco-induced immunosuppression with other immunosuppressive agents:

Immunosuppressive agents are widely used in transplant patients as well as in treatment of various diseases. It would be of interest for us to study the effect of immunosuppressive agents (i.e., Imuran, Cytoxan, Methotrexate, etc) on macrophage functions. Experiments will be devised to obtain macrophages from immunosuppressed animals and study their functional capabilities. Similar experiments could be performed on macrophages treated with immunosuppressives in vitro. Such information can be compared with our results obtained by treatment with tobacco products to arrive at a better understanding of the mechanism of action of these agents.

(3) Effect of tobacco products on macrophage participation in the immune response

It has been shown that both free macrophage-processed antigen and antigen bound to macrophage membranes after processing can be immunogenic (58-60) both in vivo and in vitro. The mode of presentation of antigen appears to be a function of the chemical and physical nature of the antigen. The initial phase of this research proposal will provide us with basic information as to the role of different physical forms of antigen in macrophage processing and therefore we can then proceed to study the effect of tobacco products on the immune response. As stated previously, several systems will be used to study these effects; we will study the primary immune response in vivo, the secondary immune response both in vivo and in vitro, and the cellular immune response.

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(a) Primary immune response in vivo:

Both alveolar and peritoneal macrophages will be prepared as described above for in vitro exposure to tobacco products. In one series of experiments, macrophages will be exposed to antigen before they are treated with tobacco products, while in another series, the cells will be treated with the tobacco products before allowed to react with antigen. After various periods of incubation, the macrophages will be collected, washed to remove free antigen, and injected into nonimmune syngeneic animals. Circulating levels of antibody will be measured by the described procedures after collection of serum on various days following primary immunization. Comparison will be made with control animals which will be injected with antigen-treated macrophages which were not exposed to tobacco products. Similar experiments will be carried out with alveolar macrophages obtained from animals exposed to tobacco products in vivo.

(b) Secondary immune response

(1) In vivo

Animals will be injected with a primary dose of KLH in vivo. Macrophages from nonimmune animals will be exposed to tobacco products and antigen as described above and then injected into the primed animals. The levels of circulating antibody will be measured one week after the animals are given the secondary injection. To determine whether macrophage-associated antigen can prime animals for a secondary response, we will inject macrophages incubated with antigen either with or without treatment by tobacco products and then challenge with free KLH. Serum will again be collected and assayed for antibody levels.

(2) In vitro

Lymph nodes and spleens from primed animals will be removed and macrophages will be separated from the population as described. These macrophages will be treated with tobacco products and antigen and then added back to the lymphocyte population. Antibody formation will be determined by the coprecipitation method and by plaque assay. In addition we will determine the effect of tobacco products on the ability of purified preparation of alveolar macrophages from nonimmune animals to present antigen to primed lymphocytes in this system.

(c) Cell-mediated immunity

Unanue and Feldman (61) have shown that in injection of macrophage-bound antigen can induce a state of delayed sensitivity in animals. Alveolar and peritoneal macrophages will be harvested from nonimmune animals and incubated with KLH. After washing, the antigen-containing macrophages will be injected alone or in Freund's complete adjuvant into the foot pad. Two to four weeks later the animal will be challenged with soluble antigen injected into the skin. The degree of erythema and induration will be measured some 24-48 hours later. These experiments will then be repeated with macrophages fron animals exposed to tobacco products in vivo as well as with macrophages treated in vitro.

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Thus, the above studies should provide us with some clear cut information concerning the role of tobacco products on macrophage function.

E. Significance of the Proposed Research

It is well appreciated that the normal functions of the immune system protect the indivudal from constant challenge by foreign materials. A breakdown of immune function, whether under natural circumstances (immunological deficiency disease and aging) or intentionally induced (treatment with immunosuppressive agents) renders the individual subject to infection by microorganisms and prone to the development of various forms of cancer.

A potentially essential link in the chain of events leading to the initiation of both the humoral and cellular immune response is the interaction of antigen with macrophages. It is suggested that this interaction results in the processing of antigen thus rendering it immunogenic. Treatment of macrophages with various agents known to interfere with their phagocytic function results in a suppressed immune response. The proposed research should generate information which may provide insight into the possible means by which tobacco products may affect macrophage function. To date, there have been no systematic approaches to the study of the effect of tobacco products on macrophages with respect to their participation in the immune response. Since alveolar macrophages play an important role in the maintenance of lung stability, the proposed research is of direct significance to the understanding of the relationship between tobacco smoke and chronic lung disease.

In addition, through the proposed research, we hope to elucidate the mechanism of antigen recognition. If we can determine which physical forms of antigen are processed by macrophages, we may acquire a better understanding of why certain antigens lead to a breakdown of immune surveillance (immunological tolerance) whereas others initiate the immune response.

Lymph nodes and spleens from primed animals will be removed and macrophages will be separated from the [illegible] described. [illegible] will be [illegible] will be determined by the [illegible]

(c) [illegible]

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